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# Determination of Achatina fulica Mucus Activity on Staphylococcus aureus

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## ABSTRACT

### OBJECTIVE

This study aimed to evaluate the in vitro antimicrobial activity of Achatina fulica mucus on S. aureus and determine the minimum inhibitory concentration (MIC) of purified mucus on S. aureus ATCC 25923 and bovine intramammary isolates.

### METHODS

Mucus was purified by high-performance liquid chromatography, and the active fraction underwent protein quantification and broth microdilution.

### RESULTS

The MIC for ATCC 25923 was 50 µg/mL; for the isolates, it ranged from 12.5 to 100 µg/mL. Two isolates (6.7%) showed MIC of 12.5 µg/mL, three (10%) 25 µg/mL, twenty-three (76.6%) 50 µg/mL, and two (6.7%) 100 µg/mL. $MIC_{50}$ and $MIC_{90}$ were 50 µg/mL and 100 µg/mL, respectively.

### CONCLUSION

The purified mucus demonstrated antimicrobial activity with bactericidal action. These findings highlight its biopharmaceutical potential for future veterinary therapy against S. aureus-induced intramammary infections.

### KEYWORDS

Achatina Fulica; Antibiotic Therapy Antimicrobial Minimum Inhibitory Concentration Bovine Mastitis; Staphylococcus Aureus.

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## INTRODUCTION

Bovine mastitis is considered the disease that causes the greatest damage to the dairy industry worldwide, as it increases production costs by reducing manufacturing yield, product quality, and shelf life. In Brazil, it is one of the main diseases affecting dairy productivity, with a high prevalence of subclinical forms that reflect significant epidemiological challenges related to management and hygienic-sanitary control. In the state of São Paulo, more than 60% of dairy cows may be affected, highlighting the need for continuous monitoring and preventive strategies. The disease leads to substantial economic losses due to reduced milk production and quality, increased treatment costs, and milk disposal, with annual losses estimated at R$ 2.8 billion.[1-5]

Staphylococcus aureus, the contagious pathogen most frequently isolates from milk of cows with mastitis,[6-14] has great epidemiological and clinical importance in mastitis associated with failures in milking management, in the prevention and diagnosis of contagious mastitis. [15-17] Researches related to the susceptibility of pathogens that cause bovine mastitis to antimicrobials demonstrates an increasing growth in the pattern of resistance, mainly for S. aureus. [8,18,19]

The evolution of S. aureus resistance to several antimicrobials raises concern. [19,20] The continuous use of these drugs for a long period, as well as their administration in high doses, represent a potential risk, as they may promote the selection of multidrug-resistant bacteria and increase the amount of residues in milk. [21,22] Natural products derived from the Brazilian fauna, especially poisons and skin secretions, are rich sources of new chemical molecules, which can be used as pharmaceutical prototypes in the development of new medications. [23]

Terrestrial molluscs Achatina fulica have antimicrobial activity on Gram positive bacteria (Bacillus subtilis and Staphylococcus spp.) and on Gram negative bacteria (Escherichia coli and Pseudomonas aeruginosa) in their skin mucus. This antimicrobial activity is attributed to achacin, a glycoprotein presents in the mucus of these animals, which mechanism of action corresponds to the inhibition of peptide glycan synthesis on the bacterial cell surface with bactericidal action. [24-26]

National researches by Martins et al. (2000, 2003)[27,28] and Lorenzi (2008), [29] demonstrated that A. fulica mucus accelerated the healing process of surgical wounds and, thus, prevented subsequent complications such as subsequent infections. Therefore, the objective of this study was to evaluate the in vitro antimicrobial activity of fulic Achatina mucus on *Staphylococcus* aureus in ATCC 252923 strain and in isolates of bovine intramammary infections.

## MATERIAL AND METHODS

### Study design and ethical considerations

This is an experimental study that was previously approved by the Ethic Committee in the use of animals of the School of Veterinary Medicine and Animal Science of University of São Paulo, protocol number 2246/2011.

### Creation and maintenance of A. fulica molluscs

The animals (n= 30) selected for mucus collection came from the Laboratory of Teaching, Research and Extension in Heliciculture and Zootherapy of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, Pirassununga-SP. The animals were separated and kept in wooden boxes measuring 65cm in length by 40 cm in width and 30 cm in height, the population density corresponded to 10 animals per box. The animals received base feed[30] and water ad libitum.

### Collection and preparation of A. fulica molt

The extraction of mucus from the animals was performed by manual stimulation of the body surface, more specifically the foot gland, not requiring sacrifice or any procedure that would cause injury to the animals studied according to the methodology described by Martins et al. (2003). [28]

The collected mucus was homogenized and divided into aliquots in sterile vials, one aliquot was diluted in 0.8% saline solution at the concentrations: 1:1, 1:2 and 1:3. Both, in natura (pure) and diluted mucus aliquots, were kept under refrigeration until the time of use.

### Purification of mucus from A. Fulica molluscs

Refrigerated in natura mucus was diluted 1:2 in Milli-Q water, the solution was homogenized and kept under refrigeration for 10 hours. Subsequently, 210 mL of ethanol were added to the solution, the solution was homogenized and cooled for another 4 hours. The solution was centrifuged at 2900 g for 30 minutes. The supernatant was discarded, and the pellet was resuspended in 20 mL in TRIS-HCl pH 7.5. Mucus purification was performed by high preparative liquid chromatography. [25]

An aliquot of the antigenic fractions was used to evaluate the protein concentration using DC Protein Assay kit (BioRad Laboratories, Hercules, CA, USA).[31] In parallel, 1D polyacrylamide gel electrophoresis was performed under denaturing conditions (SDS-PAGE) for the soluble and membrane antigenic fractions. To determine the band profile, approximately 20g/mL per cm of gel of the antigenic fractions were subjected to 1D electrophoresis. [32]

The 12% polyacrylamide gel was prepared on vertical mini-gel support. Samples were resuspended in sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 50 mM DTT, 0.01% Bromophenol Blue) and heated at 100 ºC in water bath for five minutes. After the addition of the running buffer (25 mM Tris, 192 mM Glycine, 3.5 mM SDS) electrophoresis was performed at 20 mA/gel for approximately two hours. Molecular mass standard (10 to 260 kDa - BioRad) was used to determine the relative protein bands.

### Preparation of the inoculum of S. aureus ATCC 25923

S. aureus ATCC 25923 (American Type Culture Collection, Manassas, VA, USA) was used to evaluate antimicrobial activity. For this purpose, 100 µL of suspension in glycerol containing the standard strain was inoculated into 6 mL of brain and heart infusion broth (BHI) (Oxoid, England),and incubated at 37ºC for 15 hours. After the confirmation of the growth by turbidity mean, the standard inoculum was adjusted according to the McFarland standard of 0.5 (equivalent to 5 x $10^5$ CFU/mL) used within 15 minutes according to criteria described in CLSI (2008). [33]

### Evaluation of in vitro antimicrobial activity of mucus, in natura, diluted and purified on S. aureus ATCC 25923 strain by disk-diffusion on agar

Filter paper discs with 5 mm of diameter were autoclaved and impregnated with 50 mL of diluted or purified in natura mucus. The dry disks impregnated with the active ingredients were applied to the surface of Müller Hinton agar plates (Oxoid, England) seeded with resuspended S. aureus ATCC 25923 and their concentration adjusted according to the McFarland standard of 0.5, in duplicate. As a positive control, the gentamicin disk was used, which inhibition halo for the bacterium in question is known, also in duplicate and in separate Petri plates.

Each disc was applied against the plate, to ensure complete contact with the surface of the distributed agar. The plates were inverted and placed in an oven at 35 °C for up to 10 minutes of disc application. After 18 hours of incubation the plates were removed from the oven, and the diameters of the inhibition halos were measured. Halos were measured in millimeters using a caliper according to CLSI (2008)[33] criteria.

The inhibition halo was considered the area without bacterial growth detectable to the naked eye. Halos with a diameter greater than or equal to 8 mm were considered sensitive according to the methodology described in CLSI (2008). [33]

### Determination and evaluation of Minimum Inhibitory Concentration (MIC) of Purified Mucus on S. aureus ATCC 25923 strain by microdilution in purified mucus broth

A sterile U-bottom microplate containing 96 holes was used 100 to 0.04 µg/mL were added to 100 µL of purified mucus and then 100 µL of S. aureus ATCC 25923 culture in each well. Then, the microplate was incubated in a bacteriological oven for 24 hours at 37°C. For reading, performed visually, 50 µL of tetraphenyltetrazolic acid (TTC) were added to each orifice, an indicator of bacterial multiplication, which presents reddish staining in the presence of viable cells.

To evaluate the antimicrobial activity of purified mucus, in bactericidal or bacteriostatic, an aliquot was removed from the material contained in the hole that demonstrated inhibition in bacterial development for S. aureus ATCC 25923 strain in the broth microdilution method and seeded in a sterile petri plate containing BHI agar culture mean (Oxoid, England).[34]

After incubation in a bacteriological oven for 24 hours at 37°C, the sown aliquot was visually inspected, and the result interpreted as follows: bacterial multiplication meant bacteriostatic action and absence of bacterial development meant bactericidal action.

**Determination and evaluation of the Minimum Inhibitory Concentration (MIC) of purified mucus on S. aureus isolates from bovine intramammary infections**

**Inoculum preparation**

Thirty isolates of S. aureus from bovine intramammary infections kept in the culture bank of the Milk Microbiology Laboratory of the Department of Animal Nutrition and Production of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, Pirassununga-SP were used.

For this purpose, 100 µL of each suspension in glycerol containing the isolates were inoculated into 6 mL of brain and heart infusion broth (BHI) (Oxoid, England), and incubated at 37ºC for 15 hours. After growth confirmation by turbidity of the mean, the 30 inoculum were adjusted according to the McFarland standard of 0.5 (equivalent to 5 x $10^5$ CFU/mL) to be used within 15 minutes according to the methodology described in CLSI (2008). [33]

**Microdilution method in purified mucus broth**

A sterile U-bottom microplate containing 96 holes was used. 100 to 0.04 µg/mL were added to 100 µL of purified mucus and 100 µL of each culture of the 30 S. aureus isolates from bovine intramammary infections were added to each hole. As a control of mucus dilutions, a hole containing mucus-free broth was inoculated with S. aureus ATCC 25923. Then, the microplate was incubated in a bacteriological oven for 24 hours at 37°C. For reading, performed visually, 50 µL of tetraphenyltetrazolic acid (TTC) were added to each orifice, an indicator of bacterial multiplication, which presents reddish staining in the presence of viable cells.

To evaluate the antimicrobial activity of purified mucus, in bactericidal or bacteriostatic, aliquots of the materials contained in the holes that demonstrated inhibition of bacterial development were removed in the MIC method and seeded in sterile petri plates containing BHI agar culture mean (Oxoid, England). After incubation in a bacteriological oven for 24 hours, at 37°C, the cultures referring to S. aureus isolates from bovine intramammary infections were visually inspected and the results were interpreted according to the aforementioned methodology.

**RESULTS**

**Protein quantification of purified mucus with antimicrobial activity and polyacrylamide gel electrophoresis**

The purified mucus was dosed, and a determination of 100 µg/mL was found. Polyacrylamide gel electrophoresis (SDS-PAGE) 12% indicated the presence of two protein fractions: 60 and 70 kDa (Figure 1).

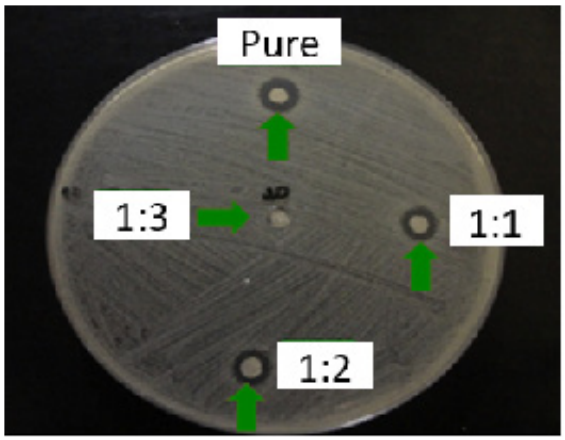
**Figure 1 -** Electrophoretic characterization in SDS-PAGE of purified mucus from A. fulica.


**Source:** The authors

**Evaluation of in vitro antimicrobial activity of mucus, in natura and diluted, on S. aureus ATCC 25923 - agar diffusion--disk technique**

In the agar disk-diffusion technique, it was observed that in natura mucus (pure) and diluted at 1:1 and 1:2 concentrations, produced inhibition halos for S. aureus ATCC 25923 strain with diameters respectively equal to 12, 10 and 8 mm, while at 1:3 concentration there was no formation of inhibition halo (Figure 2).

**Figure 2 -** Mucus agar disc diffusion, in natura (pure) and diluted 1:1, 1:2 and 1:3, on S. aureus ATCC25923 strain.

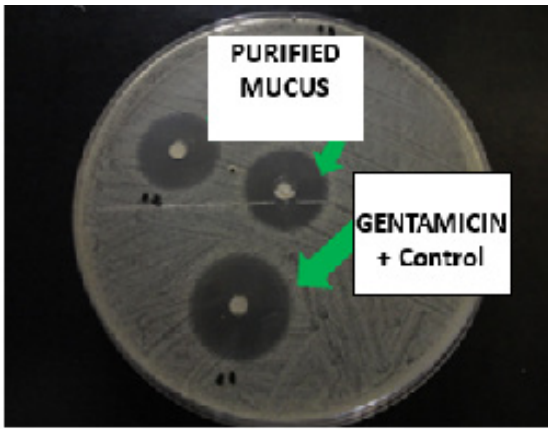



**Source:** The authors

**Evaluation of in vitro antimicrobial activity of purified mucus on s. Aureus ATCC 25923 - agar disk-diffusion technique**

Through the agar disk-diffusion technique, the production of 20 mm inhibition halos was observed for S. aureus ATCC 25923 strain (Figure 3).

**Figure 3 -** Agar disk diffusion of purified mucus and gentamicin (positive control), on S. aureus ATCC 25923 strain.




**Source:** The authors

**Determination and evaluation of MIC of purified mucus on S.aureus ATCC 25923**

The MIC of the purified mucus on S. aureus ATCC 25923 determined by the broth microdilution technique corresponded to 50 µg/mL, a value obtained by visual reading using the tetraphenyltetrazolic developer. From the broth microdilution method, the sample from the MIC determined and seeded in culture mean demonstrated that the antimicrobial activity of the purified mucus showed bactericidal action.

**Determination and evaluation of MIC of purified mucus on S.aureus isolates from bovine intramammary infections**

The MICs determined for the 30 isolates of S. aureus from bovine intramammary infections ranged between 12.5 and 100 µg/mL. Of these, two isolates (6.7%) had MIC of 12.5 µg/mL, three (10%) had MIC of 25 µg/mL, twenty-three (76.6%)

had MIC of 50 µg/mL, and two (6.7%) had MIC of 100 µg/mL (Table 1).

From the broth microdilution method, samples from the MICs determined and seeded in culture mean demonstrated that the antimicrobial activity of the purified mucus showed bactericidal action for all 30 isolates.

Through analysis, it was possible to determine MIC50, 50 µg/mL and MIC90, 100 µg/mL, confirming the effective antimicrobial activity of purified mucus on S. aureus isolates.

**Table 1 -** Frequency of S. aureus isolates from bovine intramammary infections, and their minimum inhibitory concentrations determined

| MIC (mg/mL) | Isolates from *Staphylococcus aureus* n (%) |
|---|---|
| 12.5 | 2 (6.7) |
| 25 | 3 (10) |
| 50 | 23 (76.6) |
| 100 | 2 (6.7) |

**Source:** The authors

## DISCUSSION

Adequate treatment of bovine mastitis is one of the most practical and efficient forms of control, as it eliminates an important link in the epidemiological chain of this disease.[12-14] This important infection causes significant damage to the health of dairy cows, potentially impacting the dairy cattle market. Antimicrobial susceptibility testing may support the veterinary in selecting the appropriate antimicrobial agent for the treatment of intramammary infections caused by S. aureus. [1]

There is limited evidence in the literature regarding the use of a biopharmaceutical derived from Achatina fulica, a mollusk whose mucus exhibits antimicrobial activity. A glycoprotein called achacin, composed of two subunits with molecular weights corresponding to 70 and 80 KDa, present in the mucus of these mollusks, is responsible for determining its antimicrobial activity.[35] The mechanism of action of this glycoprotein corresponds to the inhibition of peptide glycan synthesis on the bacterial cell surface with bactericidal action. [26] The results of this study corroborate the literature, since after protein isolation and purification, two proteins in the range between 60 and 70KDa were identified.

Different authors have studied the MIC determination of commercial antimicrobials, plant and propolis extracts on S. aureus. [1,34,36-38] However, MIC determination of A. fulica mucus on S. aureus isolates from bovine intramammary infections is reported for the first time in this study.

The results obtained in this study related to in vitro antimicrobial activity of A. fulica mucus, in natura and diluted, on S. aureus ATCC 25923 demonstrated a proportional decrease in the diameter of bacterial growth inhibition halos as mucus concentration was reduced.

The observation of the antimicrobial activity of mucus, both in natura and purified, on S. aureus ATCC 25923, using the agar disk-diffusion method, corroborates those described in the literature.[24,25]

Comparing the antimicrobial activity of the mucus, in natura and purified, by measuring the growth inhibition halos of S. aureus ATCC 25923, it was observed that the purified mucus showed a more effective antimicrobial activity, probably because the purified mucus presents higher protein concentration after high performance liquid chromatography. However, the use of in natura mucus may represent an alternative and low-cost source for the treatment of herds infected with S. aureus, given the high cost for protein purification.

The antimicrobial activity observed, for each MIC determined, showed bactericidal action, both for the ATCC 25923 strain and for S. aureus isolates from bovine intramammary infections, results that corroborate what was observed. [35]

From these in vitro microbiological assays, the antimicrobial activity of purified mucus was evidenced, suggesting satisfactory characteristics and with potentiality in the treatment of intramammary infections caused by S. aureus. It is imperative to consider studies to evaluate the performance of the antimicrobial activity of purified A. fulica mucus in vivo, since studies have proven the absence of its cytotoxic effect. The microbiological parameters of the Achatina fulica mucus contemplated in this study provide information about the existence of its in vitro antimicrobial activity on S. aureus.

## CONCLUSION

Purified mucus performed a more effective antimicrobial activity compared to in natura mucus. The antimicrobial activity observed, for each MIC determined with the purified mucus, showed bactericidal action for both, for S. aureus ATCC 25923 strain and for the isolates of S. aureus from bovine intramammary infections.

Therefore, the results presented in the present study allow a greater elucidation and optimization of microbiological protocols of this potential biopharmaceutical aiming its use in veterinary therapy for the control of bovine mastitis caused by S. aureus, contributing to the development of more effective and less toxic substances in the treatment and prevention of resistant microorganisms, in addition to providing atoxicity of residual effects of paramount importance for the health of the animal and human being.

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